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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The long-term **purpose** of the present proposal aims at establishing the role of the elusive mammalian protein, phosphatidycholine-specific phospholipase C (PC-PLC) in the inflammatory processes involved in progression of rheumatoid arthritis (RA).

Thus, the main **scopes** of this proposal are: 1. to identify the PC-PLC gene and protein; and 2. to test PC-PLC involvement in production of TNF alpha by monocytes.

Major findings. In these six months we have mostly worked toward the identification of PC-PLC (Aim 1). Similarly to the results we reported in the previous technical report for LPS, we found that also treatment with oxidized LDLs does not cause a reproducible activation of PC-PLC in HUVEC cells (contrary to results published by another group). Thus we have proceeded with the isolation and quality control for total RNA isolated from control HUVECs cells and from HUVECs stimulated with 20% serum at two different time points (conditions in which we have confirmed time and time again activation of PC-PLC) and in duplicate. If the quality control shows acceptable purity of RNA, we will proceed with collecting another set of samples and perform transcriptome analysis.

15. SUBJECT TERMS
Phosphatidycholine-specific phospholipase C, lipopolisaccharide, oxidized lipoproteins, serum, rheumatoid arthritis, transcriptome sequencing, HUVECs, U937 cells

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Table of Contents

Page

1. INTRODUCTION:

The present proposal aims at identifying novel players that are critically involved in the progression of rheumatoid arthritis (RA). The identification of these factors may ultimately provide alternative "druggable" targets for the treatment of this debilitating disease. The specific hypothesis that is addressed by this proposal is to test whether a mammalian protein called phosphatidycholine-specific phospholipase C (PC-PLC) might represent such a novel target. Since the mammalian PC-PLC gene has not been identified yet nor the PC-PLC protein isolated the main goals of this proposal are: 1. To identify the PC-PLC gene and protein; and 2. To test PC-PLC involvement in production of TNF alpha by monocytes.

2. KEYWORDS:

Phosphatidycholine-specific phospholipase C, lipopolisaccharide, oxidized lipoproteins, serum, rheumatoid arthritis, transcriptome sequencing, HUVECs, U937 cells.

3. OVERALL PROJECT SUMMARY:

Summary of Current Objectives.

Task 1. To identify mammalian PC-PLC

Subtask 1a: To perform whole transcriptome sequencing to identify unknown messages that are up-regulated in conditions (discovered by us in the previous months) shown to increase PC-PLC activity (serum stimulation).

As discussed in part in the first annual progress report submitted in July 2014, the conditions described in the literature by other groups and expected to increase mammalian PC-PLC (oxidized LDL and LPS stimulation) (Zhang *et al.*, 2011, Zhang *et al.*, 2010) were not reproducible and therefore could not be used as originally proposed.

Importantly, in the course of our studies, we have discovered different experimental conditions (such as serum stimulation of HUVEC cells) that lead to activation of PC-PLC, and we have defined the exact time points after serum stimulation in which elevation of PC-PLC can be detected.

We have isolated total RNA from two independent experiments in which HUVEC cells were stimulated with 20% serum and at two different time points for each experiment. We are currently testing the quality of the RNA to make sure it is compatible with transcriptome sequencing at the New York Genome Center, of which Stony Brook is one of the partners.

If the quality of RNA is acceptable a third set of samples will be prepared and the 3 sets will be sent for sequencing.

Informatics analysis of differentially expressed transcripts will be also performed by the New York Genome Center.

Up-regulated transcripts will be selected based on the following features, ordered by relevance: genes of unknown function, with one or no predicted transmembrane domains (as the results in the previous months have indicated that the PC-PLC activity that increases in response to serum stimulation resides in the cytosol) and potentially carrying one or more signature domains (C1: KxxxxxxR; C2: SGH; C3:SRxxxxxHxxxD) found in lipid phosphate phosphatases (LPPs), enzymes that cleave the phosphate group off lipids.

Subtask 1b-c: To test the identified cDNA clones for PC-PLC activity in mammalian cells.

CDNA clones corresponding to up-regulated transcripts will be then purchased from Applied Biosystems or other repositories and subcloned into a mammalian expression vector (i.e. pcDNA3.1 plasmid), transfected in Hela cells and tested for PC-PLC *in vitro* activity. Hela cells will be purchased from ATCC (with no access to identifiable information regarding the donor). The clones that confirm high PC-PLC activity as compared to the empty vector will be then down-regulated using siRNA in untransfected Hela cells (which have endogenous PC-PLC activity) to show that indeed the endogenous mammalian gene carries PC-PLC activity.

Task 2. To establish the role of PC-PLC in TNF-mediated signaling in monocytes.

Human monocytic U937 cells will be employed as experimental model and will be purchased from ATCC (with no access to identifiable information regarding the donors).

Subtask 2a: Optimization of overexpression of mammalian PC-PLC clones in U937 cells.

U937 cells will be first transfected, using the Amaxa electroporation system, with the pcDNA3.1 plasmid carrying flag-tagged mammalian PC-PLC or empty vector. After 12, 24 and 48 hours of transfection, cells will be collected and *in vitro* PC-PLC enzymatic activity will be performed (using the radioactive and fluorescence assays). At the same time points, expression of Flag-tagged PC-PLC will be confirmed by western blotting using anti-Flag antibodies (Sigma).

Subtask 2b: To test the effect of overexpressed PC-PLC on TNF signaling;

After establishing the conditions of optimal expression of PC-PLC, cells transfected with *PC-PLC* or with the empty vector will be treated with 1nM TNFα for up to 60 minutes. Cells collected at different time points (5, 15, 30, and 60 minutes) will be processed for NF-kB activation as read-out for TNFα signaling. NF-kB activation will be determined by

assessing its nuclear translocation by western blotting on nuclear versus cytoplasmic fractions and by electrophoretic mobility shift assay (EMSA) on nuclear fractions.

Subtask 2c: Optimization of down-regulation of mammalian PC-PLC clones in U937 cells.

U937 cells will be transfected using the Amaxa electroporation system with two different siRNA sequences targeting mammalian PC-PLC or a control scrambled sequence. After 12, 24 and 48 hours of transfection, cells will be collected and expression of PC-PLC mRNA will be determined by real time PCR using the expression of beta actin as normalization control. At the same time points, *in vitro* PC-PLC enzymatic activity will be performed (using the radioactive and fluorescence assays).

Subtask 2d: To test effect of down-regulation of PC-PLC on TNF signaling;

After establishing the conditions of optimal down-regulation of PC-PLC, cells transfected with two different *PC-PLC* siRNAs or with the scrambled siRNA control will be treated with 1nM TNFα for up to 60 minutes. The effect of downregulation of PC-PLC on TNF-mediated signaling will be tested on NF-kB activity and translocation as described for subtask 2b.

Summary of Results.

We have attempted to reproduce the activation of PC-PLC activity in HUVEC cells in response to oxidized-LDLs reported in the literature (Zhang *et al.*, 2011) but we found that, similarly to the LPS treatment, this activation is not reproducible and therefore cannot be utilized to identify PC-PLC. We have tested two different concentrations of oxidized LDLs in the ranges utilized in the published report (20 and 80 ug/ml) and two different time points (15 and 24 hours) (**Figure 1**).

Thus for the identification of PC-PLC we focused on the reproducible activation of PC-PLC in response to serum stimulation (20% FBS) that we have discovered and discussed in our previous technical report. We have performed the experiment two more times and side-by-side we have collected samples after 16 and 24 hours of treatment for PC-PLC activity (as positive control) **(Figure 2A and B)** and for mRNA (**Table 1**) that will be used for transcriptome sequencing. Because HUVEC cells grow on flasks that need to be coated with gelatin, the residual presence of contaminants could represent a challenge to obtain clean mRNA for transcriptome sequencing. Hence we sent the isolated mRNA collected for the experiment to check if the quality is satisfactory for transcriptome analysis. If the quality of RNA is acceptable, a third set of samples will be prepared and the 3 sets will be sent for sequencing. If not, mRNA will be isolated with an alternative method, more cumbersome but potentially more stringent.

Informatics analysis of differentially expressed transcripts will be also performed by the New York Genome Center.
Up-regulated transcripts will be selected based on the following features, ordered by relevance: genes of unknown function, with one or no predicted transmembrane domains (as the results in the previous months have indicated that the PC-PLC activity that increases in response to serum stimulation resides in the cytosol) and potentially carrying one or more signature domains (C1: KxxxxxxR; C2: SGH; C3:SRxxxxxHxxxD) found in lipid phosphate phosphatases (LPPs), enzymes that cleave the phosphate group off lipids.

Finally, as discussed earlier, we expect that the PC-PLC enzyme, given its type of biochemical reaction, will carry a signature motif found in lipid phosphate phosphatases (LPPs). Thus, as a complementary approach to the proposed transcriptome analysis, we also screened different already identified LPPs for PC-PLC activity. Briefly, we obtained constructs for the overexpression in mammalian cells of various LPPs-tagged with GFP or Myc (LPR3, LPR1, NLP1, NLP2, LPP3, ASL3, BC038108), and we transfected Hela cells with these constructs. After 24 hours from transfection, we verified their successful expression by western blotting (**Figure 3**) and collected cells and prepared total lysate for *in vitro* PC-PLC activity measured using tritiated phosphatidylcholine (PC). This method (total lysate and tritiated PC) should allow the use of less material, on the other hand, the low basal PC-PLC activity and high basal background did not allow to draw definitive conclusions. Thus we will repeat the overexpression experiment and then we will separate cytosol from membranes for each sample (vector plasmid control and LPPs plasmids) and use the fluorescent assay for PC-PLC, as we have performed for HUVEC cells. Preliminary experiments using this method have uncovered basal PC-PLC activity in Hela cells (even using as low as 20ug of proteins), and that this activity is found in the cytosol (like HUVECs) (**Figure 4**). Thus we will repeat the overexpression of LPPs and test PC-PLC activity in cytosol versus membranes fractions using the fluorescent enzymatic assay to test whether any of the already identified LPPs carries also PC-PLC activity.

4. KEY RESEARCH ACCOMPLISHMENTS:

- Preliminary results are compatible with the hypothesis that serum stimulation induces up-regulation of PC-PLC activity in HUVECs possibly through transcriptional regulation.
- Isolation of mRNA for transcriptome analysis.
- Optimized PC-PLC activity in Hela cells for overexpression of different LPPs and other candidate genes resulting from the transcriptome analysis. Of note, PC-PLC activity was detected in the cytosol (and not membrane fraction) similarly to HUVEC cells.

5. CONCLUSIONS:

Reumatoid arthritis is a significant medical challenge both in the military and general population. In fact, the limitation of physical activity or the acquisition of disability due to the disease together with high medical-related expenses (Gabriel *et al.*, 1997a, Gabriel *et al.*, 1997b) determines a poor health-related quality of life (Dominick *et al.*, 2004, Mili *et al.*, 2003).

Since RA cannot be cured, current treatments aim at reducing the chronic inflammation to slow down the disease and reduce the damage to cartilage, bone and ligaments. Current treatments for RA include non-steroidal anti-inflammatory drugs, steroids, disease-modifying antirheumatic drugs (such as the widely used methotrexane), immunosuppressants, and TNFα inhibitors (Saag *et al.*, 2008). One limitation with these treatments (in addition to the fact that they are not curative) is that they manifest serious negative side effects, such as heart problems, liver and kidney damage, increased susceptibility to infections and even increased risk of certain cancers, such as non-melanoma skin cancer (Amari *et al.*, 2011). Hence the need and interest in developing alternative treatments with a more targeted effect and less harmful side effects. One possible strategy would be to use agents with a narrower spectrum of action by blocking a specific target (i.e. PC-PLC) that acts in a specific cell type relevant to the progression of RA, such as monocytes.

The long-term objective of this proposal is indeed the investigation of whether PC-PLC might represent a safer alternative to current RA treatments. Thus the identification of PC-PLC through our proposed experiments will represent a potential new opportunity in the treatment against RA.

Importantly, the experimentation conducted so far has overcome obstacles ensuring that the proposed studies will proceed toward the identification of PC-PLC. In addition, they uncovered important new features of PC-PLC that can be instrumental in the pursue of alternative strategies and the refining of criteria for the planned experiments.

Once PC-PLC will be identified we will establish the role of PC-PLC in TNF-mediated signaling in monocytes.

6. **PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS.** Nothing to report at this time.

7. **INVENTIONS, PATENTS AND LICENCES.** Nothing to report at this time.

8. **REPORTABLE OUTCOMES**. Nothing to report at this time.

9. **OTHER ACHIEVEMENTS**. Nothing to report at this time.

10. **REFERENCES**.

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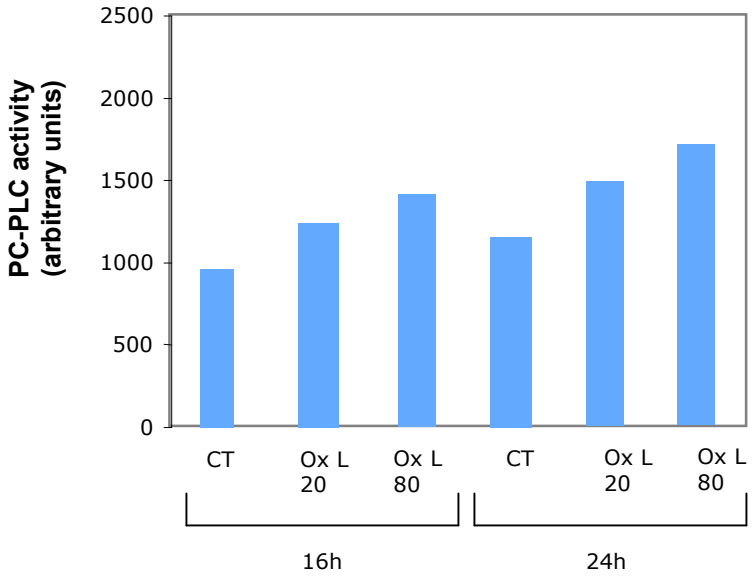

Figure 1. Ox-LDLs did not activate PC-PLC in HUVECs in a substantial manner. Serum starved HUVECs were treated with of ox-LDLs up to 24 hours. Cytosols (50ug from each condition) were prepared to measure PC-PLC activity *in vitro* and incubated for 4 hours at 37˚C.

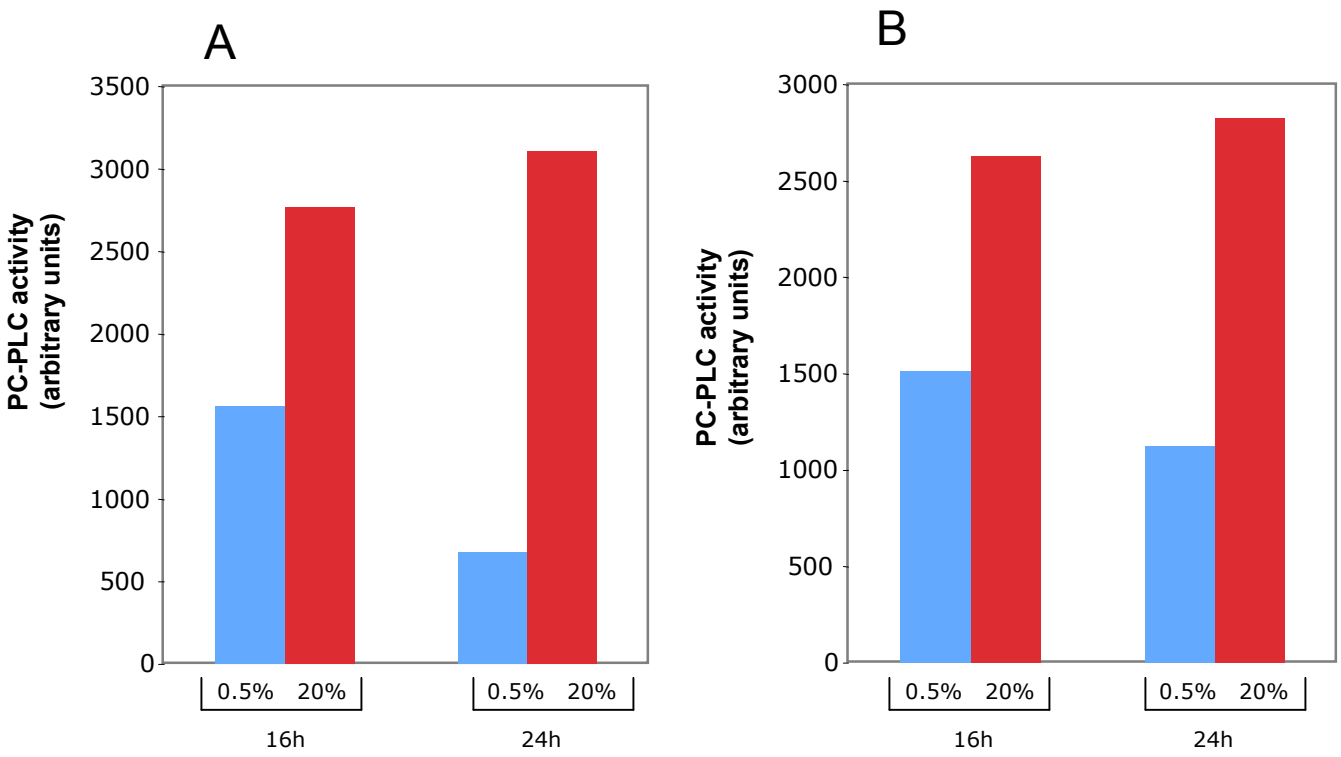

Figure 2. PC-PLC activation following serum stimulation in HUVECs. HUVEC cells were either serum starved (0.5% FBS) or serum stimulated (20% FBS). After 16 and 24hours of treatment, membranes and cytosol were prepared and PC-PLC activity was measured in cytosols. Results are representative of two independent experiments (A and B).

Table 1. Extraction of mRNA from two sets of HUVEC samples incubated with 0.5% or 20% serum and corresponding to samples for which the PC-PLC activity was shown in Figure 2.

Samples	mRNA (ug/ml)	260/280
16h, 0.5%	932.9	2.11
16h, 20%	1181.1	2.08
24h, 0.5%	901.7	2.08
24h, 20%	1124.5	2.07
16h, 0.5%	1013.1	2.08
16h, 20%	946	2.13
24h, 0.5%	717.4	2.13
24h, 20%	1244.1	2.09

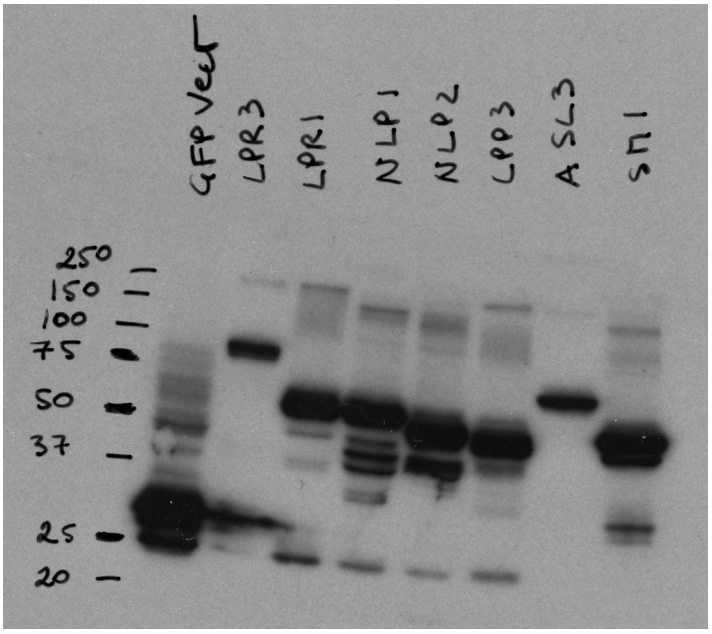

Figure 3. Transfection of different LPPs in Hela cells. Hela cells were transfected withexpression plasmids for the different LPPs tagged with GFP and after 24 hours, total proteins were collected and LPP expression was monitored by western blotting using anti-GFP antibodies. SM1 corresponds to BC038108.

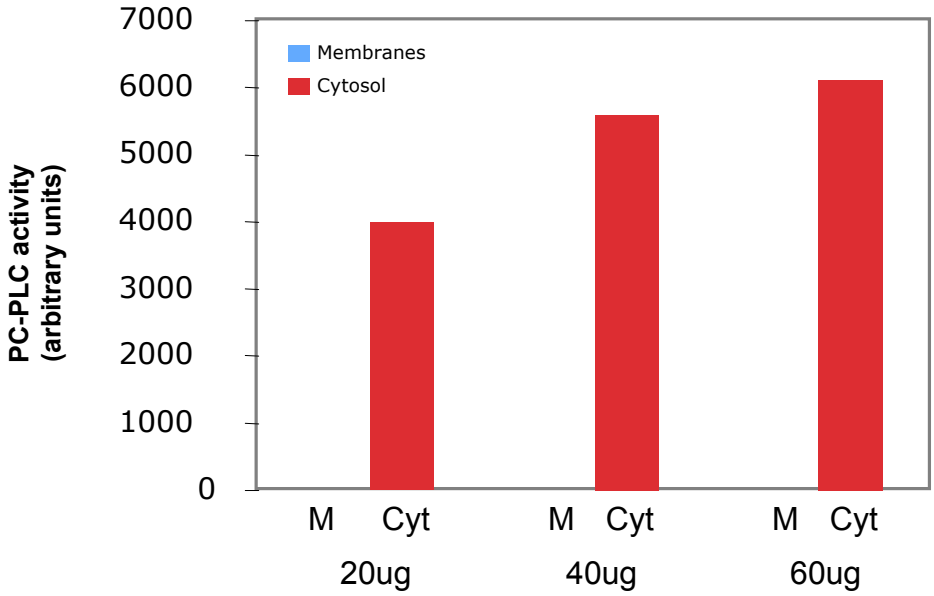

Figure 4. PC-PLC activity in vitro in Hela cells. Total lysates from control Hela cells were fractionated to separate total membranes and cytosol. Equal fractions of membranes (M) and cytosol (Cyt) (corresponding to different amounts of membranes) were compared for PC-PLC activity in vitro. Of note, PC-PLC activity in membrane fractions was undetectable.